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FINAL REPORT

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**AN ASSESSMENT OF THE PROTECTIVE EFFECTS OF
FLAX AND FLAX PRODUCTS ON THE LIVER**

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ADF GRANT
FINAL RESEARCH REPORT

for the period ending May 31, 2002

ON THE PROJECT ENTITLED :

**AN ASSESSMENT OF THE PROTECTIVE EFFECTS
OF FLAX AND FLAX PRODUCTS ON THE LIVER**

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I. AN OVERVIEW OF THE RESEARCH

1. INTRODUCTION :

1.1. FLAX :

Flax has been used for many years, by various peoples, as a health food, claimed to alleviate a variety of ailments and treat diseases including: gastrointestinal disturbances, eczema, multiple sclerosis, cancer and heart disease. As scientists have come to recognize the importance of natural products and specifically those of plant origins, flax has been begun to be subjected to scientific analysis although much is still to be done.

Flax contains phytochemicals of which two are particularly noteworthy : a 3-omega (n3) oil, alpha-linolenic acid; and products in the seed itself known as lignans, the most important of which is secoisolariciresinol (SDG).

In limited scientific studies, in animals and humans, both alpha-linolenic acid and the lignan SDG have been shown to be beneficial in modifying the expression of specific disease states, notably mammary and colon cancers, diabetes and forms of heart disease.

There are common denominators in the pathogenesis of cancer, diabetes, and heart disease that involve tissue damage through free radical production, calcium toxicity and loss of functionally critical proteins within the cell and at the level of the plasma membrane. The literature indicates that flax, flax products or and/or products comparable to flax products may be protective by working at the level of these common denominators in the case of these disease states and/or disorders.

It is significant, however, that there have been no studies on the impact of flax on liver disease even though liver disease shares with the other three disease states an involvement of free radical-induced cellular injury and toxic calcium overload. Since liver is an essential organ from a functional perspective and since liver disease is a major killer, lacks effective treatment and is increasing dramatically, studies in this area are certainly warranted.

The liver is the largest internal organ in the body and is essential to life. It develops early in gestation and performs a myriad of functions in both the fetal and adult state. The liver also performs an essential function in the metabolic breakdown and detoxification of chemical substances to achieve their elimination from the body.

Not only is the liver the site of nutrient and drug metabolism, it is sensitive to damage. Damage to this critical organ plays a critical role in the development of liver disease of which there are over 100 different forms. These invariably progress and lead to a common outcome: the development of cirrhosis of the liver and, eventually, liver cancer. The impact is highly significant in human terms. At present death by liver disease is the

fourth leading cause of death by disease in the Western World and it is increasing. Cirrhosis is associated with great morbidity and a high mortality. Liver cancer, which develops from a cirrhotic state is one of the fastest moving and lethal forms of cancer known : there is no known cure and treatments are very limited and largely ineffective. Studies are badly needed in the area of prevention and control.

1.2. FLAX AND THE LIVER

That flax may prove to be beneficial to the liver and protective against injury and/or disease is suggested from a few studies on other natural products such as the lignan TON 101 isolated from Schizandra fruit which protects against liver damage and the subsequent fibrosis associated with the development of cirrhosis of the liver induced by chronic CCl_4 treatment; and wuweizisu which protects against chemically-induced liver damage by increasing the concentration of the protective antioxidant reduced glutathione. Hepatocarcinogenesis requires, and evolves from, liver damage and liver peroxidation plays an important role in the expression of this damage. The bioflavonoid tennatin has been shown to protect against lipid peroxidation induced by the potent hepatocarcinogen aflatoxin B. Of significance here is the finding that the lignan SDG from flax has potent antioxidant properties.

Taken together, it was these results from these studies that strongly suggested that flax may also be beneficial in the treatment of liver damage and the cirrhotic and cancerous states that evolve from it.

2. OBJECTIVES :

The objectives of the our research supported by the ADF have been :

1. to definitively establish the safety of flax and flax products on the liver as it is an organ of sensitivity to being damaged.
2. to determine the impact of flax and flax products on liver damage since liver damage underscores the development of liver diseases that pose serious threats to human health
3. to identify the flax component which produces a beneficial health effect as first step in moving towards clinical trials, industrial production and therapeutic application

In order to achieve these objectives, animal models are being studied using the Fischer 344 rat which we have used extensively in the past for our studies on the liver in the normal, damaged and premalignant and malignant states.

Our experimental protocols consider these objectives and are divided into three sets of studies :

1. Developmental studies : these have been designed to establish the impact of flax feeding on the growth and development of rats and to assess their livers for indicators of hepatoprotection/toxicity.
2. Studies on the effect of flax feeding on CCl₄-induced liver damage : these have been designed to see if flax can protect against liver injury produced by this potent hepatotoxic chemical
3. Studies on the effect of flax feeding on chemically-induced liver cancer: these are designed to see if flax can prevent or treat liver cancer.

3. EXPERIMENTAL DESIGN :

3.1. DIETS :

i. **Regular Rodent chow** - PMI rodent chow will be obtained through the Animal Resource Center, University of Saskatchewan.

ii. **Flaxseed**

Norlin - a high linolenic acid flax

Low linolenic acid flax - a low linolenic acid flax.

iii. **SDG**

To make the 10% flax diets, regular chow was ground to a powder in a stainless steel animal food grinder (Department of Physiology). Flax was ground using a Proctor Silex blender. A 10% diet was then made : 1 part flax + 9 parts regular chow (v/v). This was well mixed. Sufficient water was added to re-form the chow into cylinders, comparable to those of the regular chow. These were dried at low heat for 1 hour and then given to the animals. Regular rodent chow was treated in the same way.

3.2. TREATMENT GROUPS :

In all of the studies, a common approach in assessing the impact of flax was taken as outlined. Animals from the various experimental models and were divided into the following groups :

- i. **Group I** : animals were fed regular rat chow
- ii. **Group II** : animals were fed regular rat chow containing 10% flax

3.3. PROTOCOLS :**1.1. Development** - from day one after birth to 85 days after birth**Groups :**

Regular chow fed rats : 30 - 50

Flax chow fed rats : 30 - 50

1.2. Liver Damage - from the potent hepatotoxin CCl₄**Groups :**

Regular chow fed :

- i. untreated : 20
- ii. olive oil treated controls : 20
- iii. CCl₄ treated : 20

Flax chow fed :

- i. untreated : 20
- ii. olive oil treated controls : 20
- iii. CCl₄ treated : 20

1.3. Liver Cancer - induced by the chemical carcinogen diethylnitrosamine**Groups for each of 2 protocols**

Regular chow fed rats :

- i. saline injected : 40
- ii. DENA injected : 40

Flax chow fed rats :

- i. saline injected : 40
- ii. DENA injected : 40

Protocols :

Protocol I : flax exposure AFTER DENA

Protocol II : flax exposure BEFORE DENA

II. PROGRESS FROM OUR LABORATORY :

1. DEVELOPMENTAL STUDIES

1. Our results demonstrate on short term feeding (6weeks) of flax to adult rats and gerbils that: flax chows are well tolerated and body weight gain is comparable between flax-fed and regular chow-fed groups.
2. Our results also show in progeny from mothers fed flax chow before and after birth that: developmental indices, both behavioral and physical, are indistinguishable from regular chow-fed progeny from mothers that were fed regular rat chow. The growth curves for these rats are shown in **Figure 1**.
3. Both short-term flax feeding and chronic exposure extending from before birth until adulthood :
 - a. has no effect on glucose balance since blood glucose levels are comparable between flax-fed and regular-chow fed groups
 - b. has no effect on pancreatic hormones implicated in maintaining nutrient balance
 - c. increases a liver enzyme γ -glutamyltranspeptidase (γ GT) that has been linked to increasing reduced glutathione in the liver – a protective compound that guards against liver damage; this increase is more pronounced in the male than in the female. Results from the long term exposure are shown in **Figure 2**.
4. Studies using flax differing in oil (alpha-linolenic acid : ALA) content, but not the lignan (secoisolariciresinol : SDG) content, reveal, as illustrated in **Figure 3**, that the flax-induced increase in the activity of γ -glutamyltranspeptidase is due to SDG and not ALA.
5. Both short-term flax feeding and chronic exposure extending from before birth until adulthood are without effect on plasma transaminase levels which are plasma markers of liver damage. Levels ranged from 10 – 25 SF units/ml, well within normal values.

These results show that flax feeding, and early and persistent exposure to flax, is without negative effects on the growth and development of the gerbil and the rat, and, by increasing the activity of an enzyme in the liver linked to hepatoprotection, γ -glutamyltranspeptidase (γ GT), may afford protection against damage. That the increase in the enzyme activity is greater in males than in females, suggests that the flax effect is mediated through the estrogenic lignan compound. This is confirmed in separate studies using strains of flaxseed differing in the oil but not lignan content. The liver is not damaged by flax feeding either in the short term or long term protocols since the plasma transaminase levels are unaffected.

2. STUDIES ON LIVER INJURY

While the above studies establish the biosafety of flax on the animal and at the level of the liver, and suggest that flax produces liver changes that may be hepatoprotective, the studies on CCl₄-induced injury are essential to confirm hepatoprotection.

These studies, the results of which are summarized in **Figure 4**, indicate that *flax feeding does protect the liver against CCl₄-induced liver damage* as flax-fed animals exhibit a reduction in the injury marker - plasma transaminase levels - compared to regular chow fed rats. This is a very important finding since CCl₄ is a very potent hepatotoxic agent and this degree of reduction in injury is highly significant.

We are looking at the status of the calcium pumps in the liver cells that are implicated in CCl₄-induced liver damage. This will allow us to determine which of the many calcium pumps may be the site of hepatoprotection.

The findings indicate that the calcium pump in the endoplasmic reticulum is decreased to a lesser extent by CCl₄ in the flax-fed vs the regular chow-fed rat. This may be one of the critical site of hepatoprotection in the flax-fed animal.

3. STUDIES ON CHEMICALLY-INDUCED LIVER CANCER :

Liver injury is the common denominator and central feature of the development of liver diseases including liver cancer. Although the studies on CCl₄ suggest that flax may be protective against liver disease in general because it protects against liver injury, the final series of experiments tested this hypothesis specifically by examining the impact of flax feeding on the development of liver cancer by the chemical carcinogen diethylnitrosamine.

In these studies, **two protocols** were followed.

In the first protocol, the rats were fed regular rat chow, and, at the time of weaning, were given DENA. They were then switched to flax chow or maintained on regular chow. As depicted in **Figure 5**, at 55 days of age, there was a significant DENA-induced rise in the activity of γ GT, here used as a liver cancer marker. However, the DENA-induced increase in this enzyme was very much less in rats fed flax chow. These results demonstrate that *flax consumption decreases the progression of DENA-evoked liver cancer development*.

In the second protocol, the rats were exposed to regular chow or flax chow before birth and throughout the rest of their lives. At weaning, the carcinogen DENA was administered and rats assessed at 53 days of age. As illustrated in **Figure 6**, the DENA-induced increase in γ GT was very much less in the flax chow-fed rats compared to the regular chow-fed rats. These results indicate that *flax consumption decreases the initiation and progression of liver cancer in response to DENA*.

Taken together, these important findings suggest that flax consumption can protect against both the initiation and the promotion of liver cancer.

III. FUTURE PLANS :

a. Development :

We have shown that flax exposure has no deleterious effects in first generation flax fed rats born to dams consuming flax from the 16th day of pregnancy. Since SDG is estrogenic, we would like to extend these studies to look at the second generation of rats, with special emphasis on sex hormone levels and reproductive status.

b. Injury :

We have shown that flax protects against acute liver injury in response to an hepatotoxic agent. This has implications to liver cirrhosis since cirrhosis evolves from injury and is the common end stage of liver disease. We would like to look at the development of cirrhosis, from its inception, through its progression, to its final end state in order to determine at which point(s) protection is achieved.

c. Cancer :

We have demonstrated that flax attenuates the development of the early stages of liver cancer. We would like to examine the entire course of hepatocarcinogenesis to determine the final outcome of the process and the points at which attenuation can be achieved.

d. SDG :

SDG has been implicated in our studies in producing protective hepatic effects but we have been limited in examining SDG due to its very limited availability. We would like to use an SDG enriched product to examine SDG involvement further. This product has just recently become available.

ILLUSTRATED RESULTS OF THE RESEARCH STUDIES

Figure 1 : Growth curves of regular chow fed and flax chow fed rats.

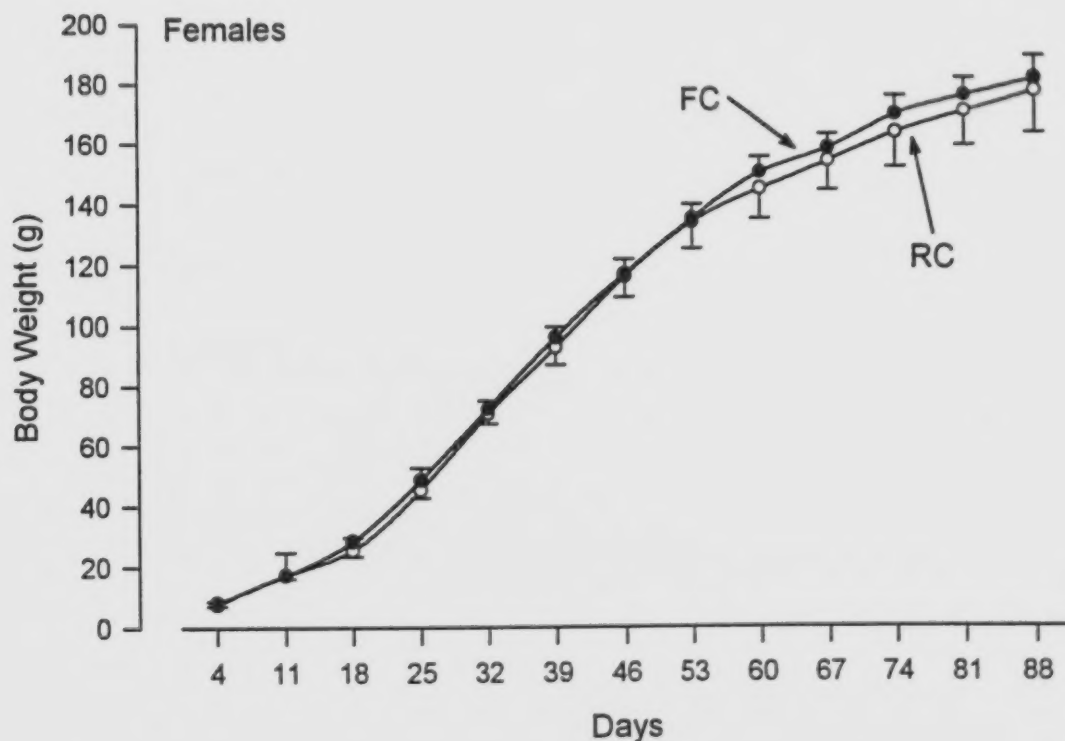
Figure 2. Development of liver γ GT in regular chow fed and flax chow fed rats.

Figure 3. The effect of Solin and Norlin flax chow on liver γ GT .

Figure 4. The impact of CCl_4 on GPT in regular chow fed and flax chow fed rats.

Figure 5. The effect of flax chow on DENA-induced liver cancer : exposure before DENA.

Figure 6. The effect of flax chow on DENA-induced liver cancer : exposure after DENA.

**FIGURE 1a.**

Timed pregnant Fischer 344 rats were placed on regular chow (RC) or 10% flax chow (FC) at the 16th day of gestation and maintained on these diets for the entire experiment. Pups born to these rats were weaned at 31 days onto the same diet consumed by the mother. Body weights of the rats were determined throughout development, from immediately postnatal to adulthood. Results represent mean values $\pm$ standard error of the mean for body weights of 5-7 rats per group.

Significance : there is no effect of flax exposure on the growth of the Fischer 344 rat.

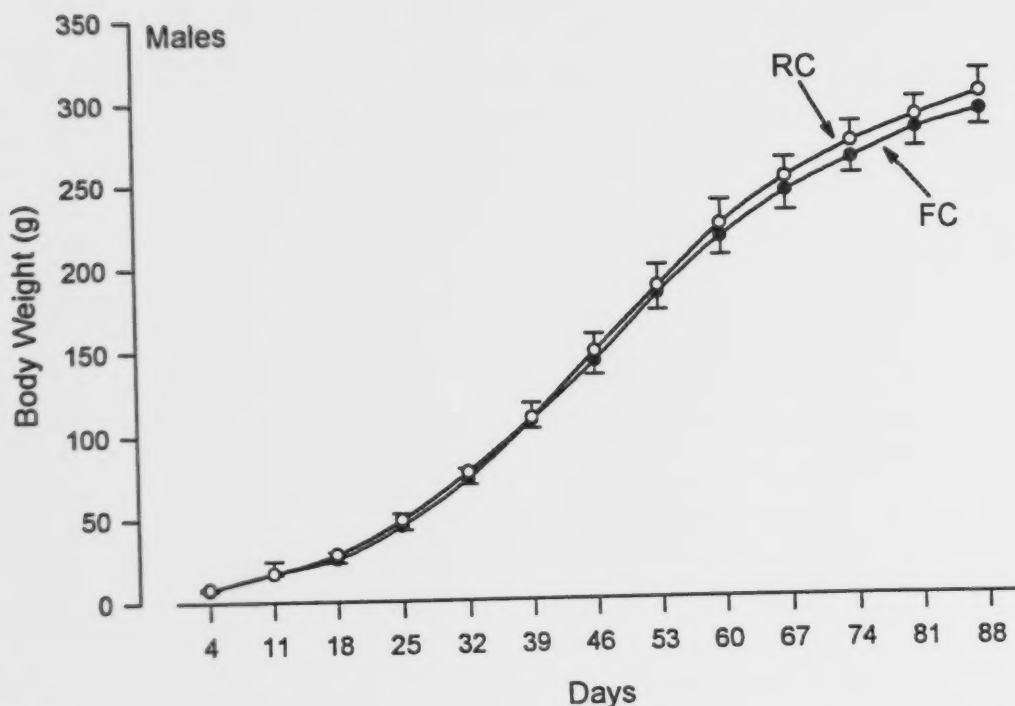


FIGURE 1b.

Timed pregnant Fischer 344 rats were placed on regular chow (RC) or 10% flax chow (FC) at the 16th day of gestation and maintained on these diets for the entire experiment. Pups born to these rats were weaned at 31 days onto the same diet consumed by the mother. Body weights of the rats were determined throughout development, from immediately postnatal to adulthood. Results represent mean values $\pm$ standard error of the mean for body weights of 5-7 rats per group.

Significance : there is no effect of flax exposure on the growth of the Fischer 344 rat.

DEVELOPMENT OF LIVER γ GT IN REGULAR CHOW AND FLAX CHOW FED RATS

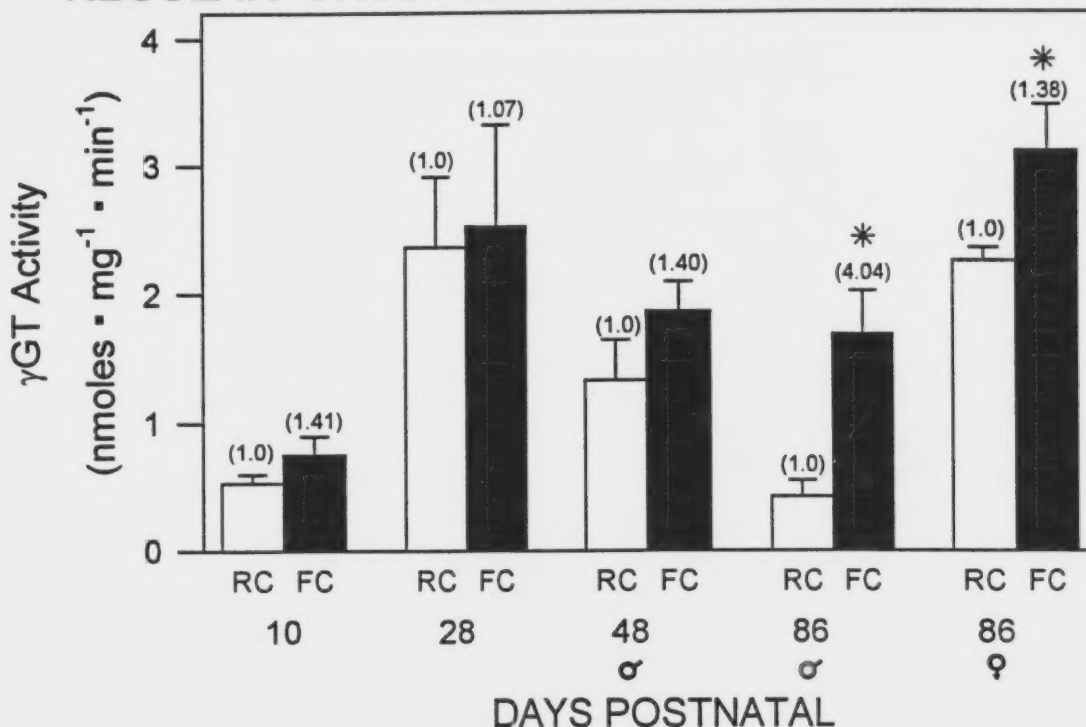


FIGURE 2.

Timed pregnant Fischer 344 rats were placed on regular chow (RC) or 10% flax chow (FC) at the 16th day of gestation and maintained on these diets for the entire experiment. Pups born to these rats were weaned at 31 days onto the same diet consumed by the mother. Rats were studied from day 10 to day 86 postnatal. At these times they were sacrificed and livers removed. Homogenates were prepared from the livers and the activity of γ -glutamyltranspeptidase (γ GT) determined. Numbers above the FC bars represent fold difference from RC, set at 1.0. Results represent mean values $\pm$ standard error of the mean for 5-7 liver homogenates, each derived from the liver of a separate rat. * indicates statistical significance set at $p < 0.05$.

SIGNIFICANCE : flax consumption leads to an increase in liver γ GT post-puberty in male and female rats, more so in the former than the latter suggestive of an involvement of the estrogenic lignan SDG.

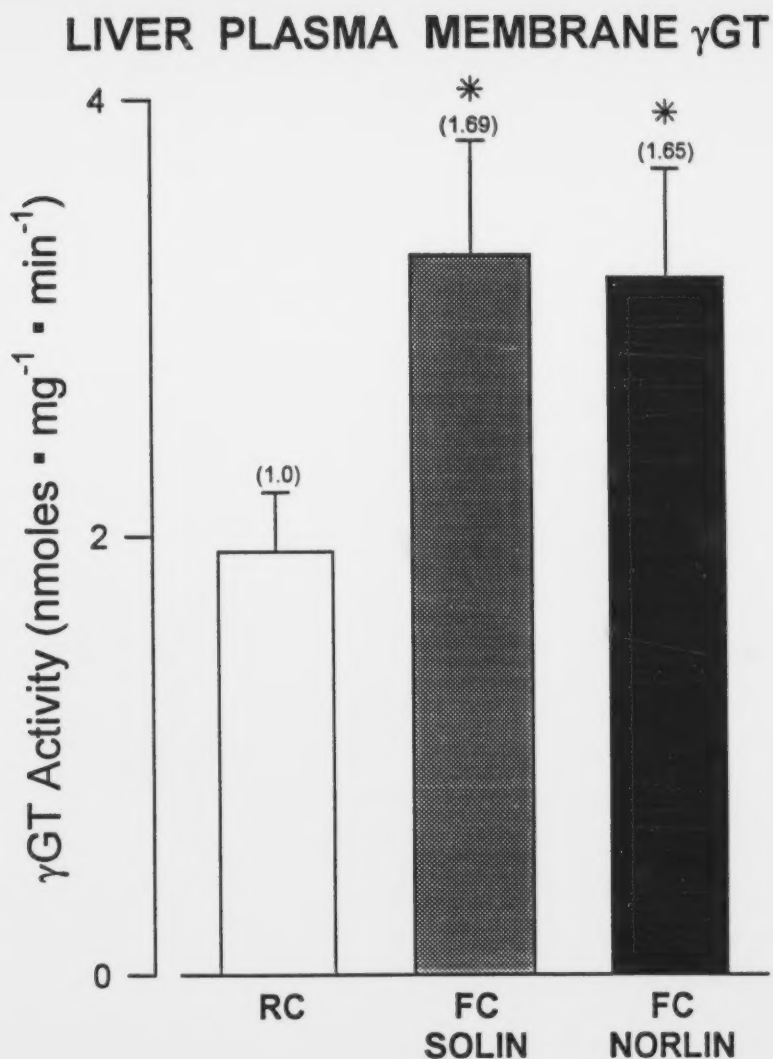
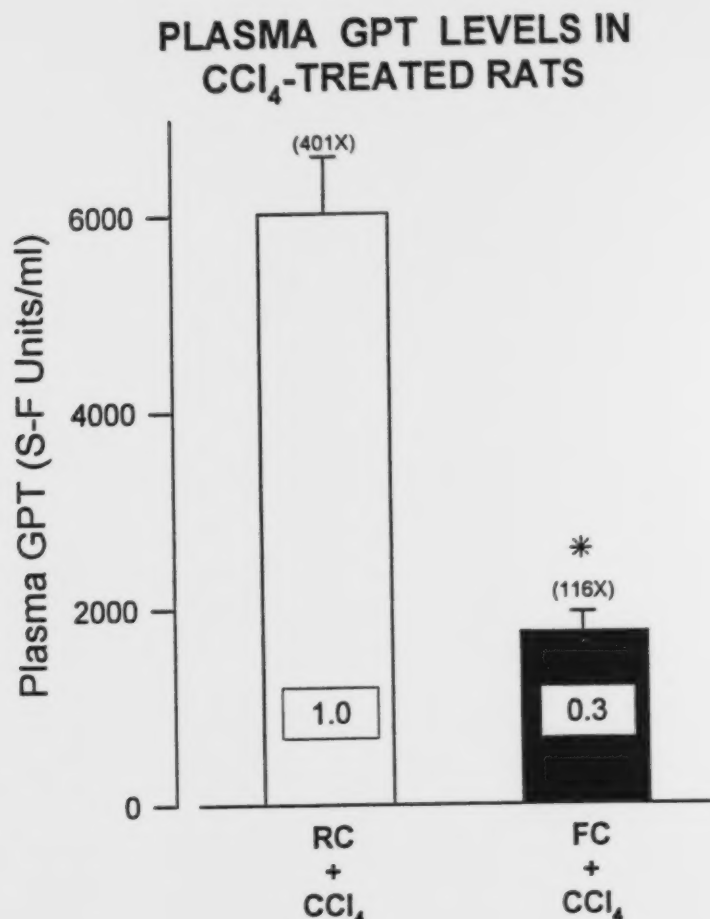


FIGURE 3.

Adult male Fischer 344 rats, 50 days of age, were fed regular chow (RC), 10% Solin flax chow (FC-SOLIN) or 10% Norlin flax chow (FC-NORLIN) for 45 days which time they were sacrificed and livers removed. Liver plasma membranes were prepared and the activity of γ -glutamyltranspeptidase (γ GT) determined. Results represent mean values $\pm$ standard error of the mean for 5-7 liver plasma membrane preparations, each derived from the liver of a separate rat. The numbers above the FC bars represent fold difference from RC, set at 1.0. * indicates statistical significance set at $p < 0.05$.

SIGNIFICANCE : SDG is implicated in the flax-induced increase in liver γ GT.

**FIGURE 4.**

Timed pregnant Fischer 344 rats were placed on regular chow (RC) or 10% flax chow (FC) at the 16th day of gestation and maintained on these diets for the entire experiment. Pups born to these rats were weaned at 30 days onto the same diet consumed by the mother. At 85 days of age, rats were given either CCl₄ by gavage at a dose of 0.25 ml of CCl₄ cut 1:1 in olive oil/100mg body weight; or, olive oil alone. 24 hours later rats were sacrificed. Whole blood was collected and plasma prepared. The level of plasma GPT was determined. Results represent mean values $\pm$ standard error of the mean for 5-7 plasma samples per group each derived from a separate rat. Data indicates the values for the CCl₄-treated regular chow-fed rats (RC : open bars) and CCl₄-treated flax chow-fed rats (FC : closed bars). Numbers above the CCl₄ bars indicate fold increase from olive oil control rats. Numbers within the closed bar, FC group, represent the fold difference in level from that in the open bar, RC group, set at 1.0. * indicates statistical significance set at $p < 0.05$.

SIGNIFICANCE : flax consumption lessens the CCl₄-induced increase in plasma GPT indicating that flax exerts a hepatoprotective effect on the liver resulting in a decrease in damage in response to injury.

EFFECT OF DIETHYLNITROSAMINE ON LIVER PM γ GT

A. DIETARY EXPOSURE AFTER DENA

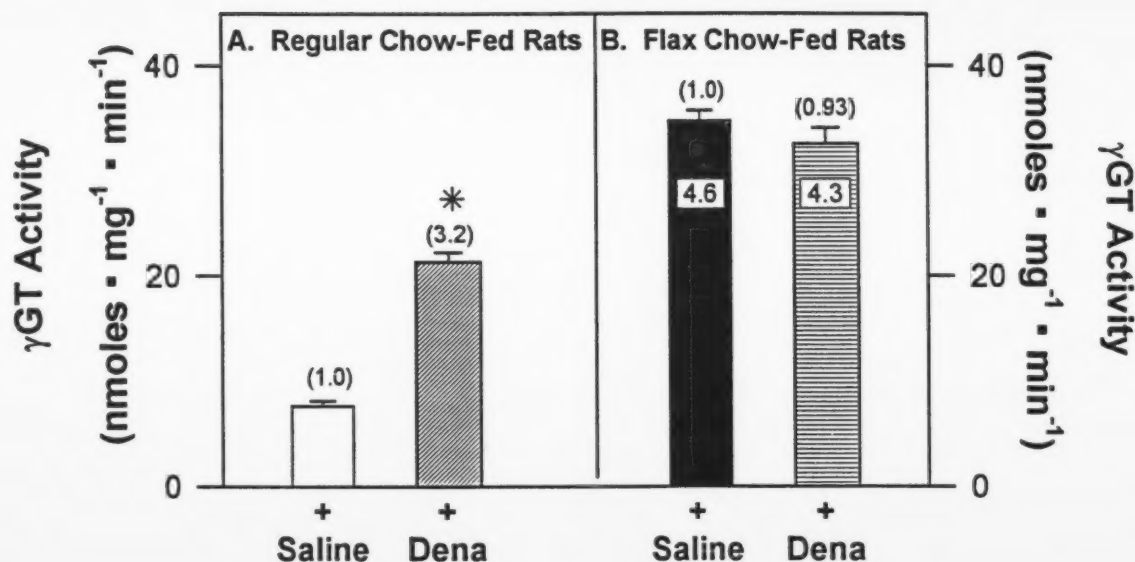


FIGURE 5.

Timed pregnant Fischer 344 rats were maintained on regular chow from the 16th day of gestation. On day 27, either saline (SALINE) or diethylnitrosamine (DENA) at a dose of 200 mg/Kg was administered. On day 28, rats were weaned onto either regular chow or 10% flax chow. At 55 days of age, rats were sacrificed and livers removed. Liver plasma membranes were isolated. The activity of γ -glutamyltranspeptidase (γ GT) was determined in plasma membranes prepared from the livers of rats belonging to the regular chow-fed (Panel A) and flax chow-fed rats (Panel B). Results represent mean values $\pm$ standard error of the mean for 3 liver plasma membrane preparations per subgroup each derived from the liver of a separate rat. Numbers above the bars represent the fold difference between activities in the DENA and SALINE groups with the latter set at 1.0. * indicates statistical significance set at $p < 0.05$.

SIGNIFICANCE : when fed after the administration of a carcinogen, flax consumption leads to a decrease in the carcinogen-evoked increase in liver γ GT, indicating that flax feeding protects against the promotion and progression of liver cancer.

EFFECT OF DIETHYLNITROSAMINE ON LIVER PM γ GT

B. DIETARY EXPOSURE BEFORE DENA

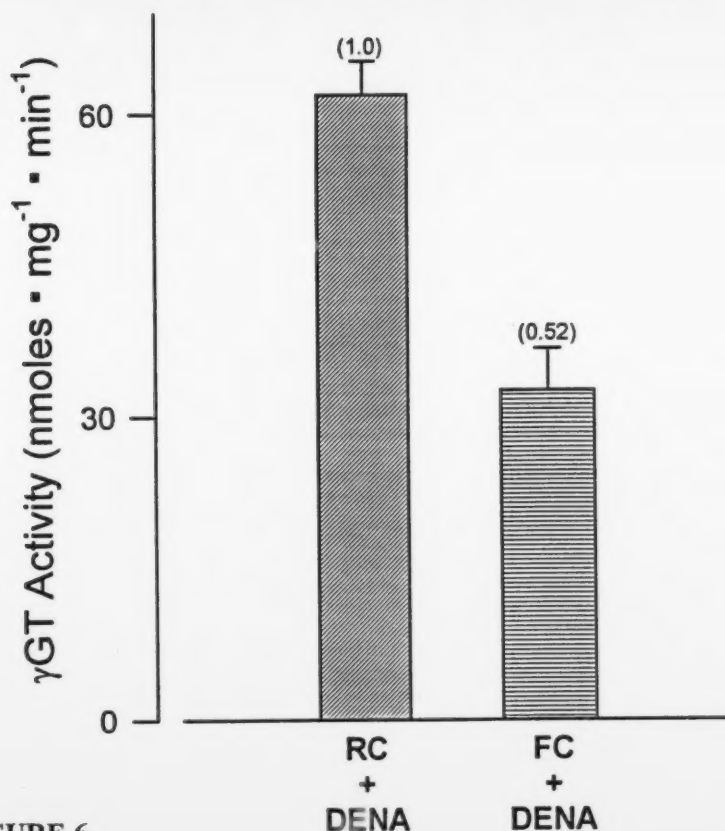


FIGURE 6.

Timed pregnant Fischer 344 rats were placed on regular chow (RC) or 10% flax chow (FC) at the 16th day of gestation and maintained on these diets for the entire experiment. On day 27, diethylnitrosamine (DENA) at a dose of 200 mg/Kg was administered to pups belonging to the regular chow-fed (RC + DENA) or flax chow-fed (FC + DENA) group. Pups were weaned at 28 days onto the same diet consumed by the mother. At 53 days of age, rats were sacrificed and livers removed. Liver plasma membranes were prepared and the activity of γ -glutamyltranspeptidase (γ GT) determined. Results represent mean values $\pm$ standard error of the mean for 3 liver plasma membrane preparations per group each derived from the liver of a separate rat. Numbers above the bars represent the fold difference between activities in the DENA and SALINE groups with the latter set at 1.0. * indicates statistical significance set at $p < 0.05$.

SIGNIFICANCE : when fed before the administration of a carcinogen, flax consumption leads to a decrease in the carcinogen-evoked increase in liver γ GT, indicating that flax feeding protects against the initiation and/or promotion and progression of liver cancer.

EXPENDITURES :

1. Material and Supplies :

These represent costs for items purchased from Medical Stores, University of Saskatchewan which offers a wide selection of competitively priced biomedical supplies. These items include general lab chemicals, glassware and plasticware necessary for carrying out the research project.

Specifically, these purchases have included :

- glass beakers and jars - assorted sizes for making up solutions and processing samples
- pipette tips - for pipettes used ion assaying samples
- Parafilm - for covering samples to be stored at -80°C
- Vacutainer tubes - for preparation of plasma from whole blood
- Test tubes - for carrying out enzyme assays
- Gloves and masks for animal handling purposes
- Miscellaneous small items such as special dishwasher soap (phosphate free)

2. Linen :

For cleaning of lab coats.

3. Printing :

These costs represent photocopying and printing of materials related to the research project : articles from the scientific literature and summaries of data.

4. Deliveries :

This amount covered delivery and courier charges.

5. Animals

This represents the ordering of and – effective – Nov 1, 2001 – the care for animals

6. VCR Canlab

This is for the purchase of laboratory supplies such as homogenizers and tubes from VCR Canlab.

7. Salaries :

*** I have a full time technician working on the ADF grant : Dr. Y Song. In addition, I have had a 3/5 time technician - Ms. Liz McLean - working on a Sask Flax Development Commission Grant which is a match grant for the ADF project. Thus, they are working

together on a jointly funded project. For purposes of accounting, Ms. McLean was paid from the ADF grant until December 1, 2001 at 3/5. Hence, the total for the salary support. The Sask Flax grant has covered animal and animal care costs as well as others.

